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14. ABSTRACT
In 30 to 70 % of systemic sclerosis patients, the disease progresses to the lungs and internal organs resulting in tissue damage and remodeling, inflammation, and fibrosis (i.e. the overexpression of collagen). Lung fibrosis is the major cause of morbidity and mortality in scleroderma and is particularly devastating because there are no FDA-approved treatments. The overexpression of collagen is accompanied by the overexpression of other extracellular matrix molecules including periostin. Periostin is of particular interest because it regulates inflammatory cell infiltration and the differentiation of fibroblasts (the cell type that expresses collagen in fibrotic tissue) in other systems. For these reasons, we are determining whether periostin plays a regulatory role in the progression of lung injury/fibrosis, possibly by regulating inflammation or possibly by regulating the differentiation of fibroblasts and their expression of collagen. Indeed, our recent experiments using periostin knockout mice validate the importance of this molecule in that lung injury/fibrosis in these mice is worse than in control mice. The results of these studies may suggest novel therapies for lung fibrosis in which the function(s) of periostin are altered.

15. SUBJECT TERMS
Systemic sclerosis, periostin, inflammation, fibroblasts, lung fibrosis, collagen, knockout mice

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INTRODUCTION

Systemic scleroderma is a debilitating disease that affects approximately 100,000 people in the US, mostly women, and is characterized by immune system activation, autoimmunity, small-vessel vasculopathy, and skin fibrosis. In 30 to 70 % of patients, the disease progresses to the lungs and other internal organs. Lung fibrosis (i.e. the excessive accumulation of extracellular matrix [ECM] proteins, particularly collagen I) is the major cause of morbidity and mortality in scleroderma. The overexpression of collagen I in fibrotic lung tissue is accompanied by the overexpression of other ECM molecules (e.g. the proteins periostin, tenascin-C, and versican and the glycosaminoglycan hyaluronic acid) and by inflammatory cell infiltration. In the simplest viewpoint, it is possible that tissue damage leads to inflammatory cell infiltration and that in turn cytokines produced by inflammatory cells and damaged tissue promote the expression of collagen I and other ECM molecules. However, it is more likely that the regulation of these processes (inflammatory cell infiltration, collagen I expression, expression of other ECM molecules) are intertwined in a complex manner. For example, periostin is known to play a critical role in regulating the differentiation of fibroblasts and the expression of collagen in the heart both in normal development and in injured adult tissue (1-4). Periostin has also been shown to regulate inflammatory cell infiltration in allergic disease in the esophagus and lung (5). Therefore, it is likely that periostin will also regulate the differentiation and function of fibroblasts/ myofibroblasts and the infiltration of inflammatory cells in fibrotic adult lung tissue. Our long-term goal is to determine how modulation of the expression and function of periostin will alter the progression of lung fibrosis by affecting fibroblast differentiation, the expression of collagen I by lung fibroblasts, and inflammatory cell infiltration. These experiments will be performed in both in vitro and in vivo model systems. In vitro experiments will be performed using cultured lung fibroblasts and will focus on cell adhesion to periostin and the ability of periostin to regulate collagen expression. In vivo experiments will be performed using mice in which lung fibrosis is induced by intraoral delivery of bleomycin and will focus on mice in which periostin expression is knocked out or in which a polypeptide equivalent to the alternatively-spliced C-terminal region of periostin is overexpressed. While bleomycin may not be a perfect model for human diseases, it is a very convenient and reliable way of inducing lung fibrosis in mice and is the best available model for scleroderma lung disease because both bleomycin treatment and scleroderma lung disease produce inflammatory alveolitis and overproduction of collagen and other ECM molecules.

BODY

Our long-term goal is to determine how modulation of the expression and function of ECM molecules alters the progression of lung fibrosis by affecting fibroblast differentiation, the expression of collagen I by lung fibroblasts, and inflammatory cell infiltration. Both the ECM protein periostin and the ECM glycosaminoglycan hyaluronic acid are molecules of interest in this regard. While this project originally focused on hyaluronic acid, we found it necessary to change our focus to periostin because extreme variability in the results of our experiments on hyaluronic acid forced us to abandon these studies. Accordingly, we submitted a revised Statement of Work which was approved. In our studies on hyaluronic acid, we tried to overcome the observed variability by using lung fibroblasts from control subjects and from scleroderma patients as well as an embryonic lung fibroblast cell line, but we repeatedly were faced with the same obstacle. We also tried to address the problem using HA oligosaccharides (to inhibit HA signaling) and intact HA (to promote HA signaling) but again to no avail. This variability may result from multiple HA signaling mechanism being operative in these cells, some of which promote collagen expression and some of which inhibit collagen expression. Thus, in one experiment, the inhibitory mechanism may be predominant while in another experiment the

positive mechanism may be predominant due to an extremely minor variation in experimental conditions that is beyond our ability to identify. While we have decided that we must abandon these in vitro experiments on HA, we still plan to perform a limited number of the experiments on HA in vivo that we had proposed in our original Statement of Work.

ECM proteins influence cell behavior by binding to specific cell-surface receptors. Specific functional domains within each ECM protein are involved in these interactions. To begin to identify functional sequences in periostin and their cell-surface receptors on lung fibroblasts, we compared the ability of lung fibroblasts to bind to a fusion protein equivalent to the alternatively-spliced C-terminal domain of periostin, full-length periostin, and fibronectin (a positive control ECM protein providing extremely strong cell adhesion). As expected, strong adhesion to fibronectin was observed which was almost completely blocked by EDTA, but not by heparin (Table 1). Good adhesion to the periostin C-terminal domain was observed which, in contrast, was completely blocked by heparin but only slightly blocked by EDTA (Table 1). Adhesion to full-length periostin was only at an unreliable low level so is not shown. The fact that adhesion to fibronectin was blocked by EDTA, but not heparin, is consistent with the idea that the cell-surface receptor for fibronectin in these cells is a member of the integrin family. The fact that adhesion to the periostin C-terminal domain was blocked by heparin, but not EDTA, strongly suggests that its receptor is a heparan sulfate proteoglycan and not an integrin. The fact that little, if any, adhesion to full-length periostin was observed cannot be interpreted until we do experiments in which equimolar solutions (rather than equal concentration solutions) of full-length periostin and periostin C-terminal domains are used to coat the plastic substratum.

Table 1. Cell Adhesion to the Periostin C-Terminal Domain

	Inhibitors		
ECM Protein Coat	None	EDTA	Heparin
Fibronectin	530 ± 81	59 ± 9	411 ± 61
Periostin C-Terminus	218 ± 85	168 ± 18	0

Non-tissue culture-treated wells of 96-well plates were coated with 10 µg per ml solutions of fibronectin, the periostin C-terminal fusion protein, or full-length periostin for 1 h, then blocked with 10 mg per ml of BSA. Lung fibroblasts (20,000 MRC-5 cells in 0.1 ml serum-free medium) were incubated in duplicate wells for 1 h, the wells washed, and bound cells stained using crystal violet. The dye was then solubilized and adhesion quantified in terms of the OD_{550} of the dye solution.

To begin to understand the role of periostin in the progression of lung fibrosis, we examined its distribution in normal lung tissue and in the lung tissue of mice in which lung injury/fibrosis had been induced using bleomycin (Fig. 1). In normal lung tissue, periostin is uniformly expressed in alveolar walls. Whether it is expressed by epithelial cells or fibroblasts remains to be determined. However, in fibrotic lung tissue periostin staining is present in an intense fibrillar pattern in the distorted portion of the lung that is filled with ECM and inflammatory cells. In contrast, in the portion of the fibrotic lung with relatively normal morphology, periostin staining in the thickened alveolar walls does not appear to be as uniform as in control alveolar walls. Therefore, the distribution of periostin in normal and fibrotic lung tissue appears to be very different.

To further understand the role of periostin in the progression of lung fibrosis, we treated control mice and periostin knockout mice with bleomycin. Twelve days later the mice were sacrificed and tissue sections and tissue extracts were prepared. Based on results in the heart (1-4), we thought it likely that the presence of periostin would be correlated with higher levels of lung fibrosis. However, this was not the case. The morphology of bleomycin-treated lung tissue

was noticeably worse in the knockout mice than in the control mice (Fig. 2 A, B). The difference between knockout and control mice was even more apparent in Western blots of tissue extracts (Fig. 2C). Knockout mice showed considerably higher levels of tenascin-C and of collagen expression (detected using an antibody against the pro-domain of the collagen I α1 subunit which recognizes collagen molecules that are still associated with the cell in which were synthesized and have not yet been incorporated into the ECM). These results strongly suggest that the mechanism of action of periostin in lung fibrosis differs from its mechanism of action in heart disease. There are many ways that this could occur. For example, myocardial cells are major players in the heart but are not present in the lung. On the other hand, most of the cells in the normal lung are epithelial cells while there are few of these cells in the heart. Therefore, the distinctive functions of periostin in the heart and lung may depend on its roles in myocardial and epithelial cell biology. Alternatively, the role of periostin in regulating the behavior of inflammatory cells may be very different in the heart and the lung.

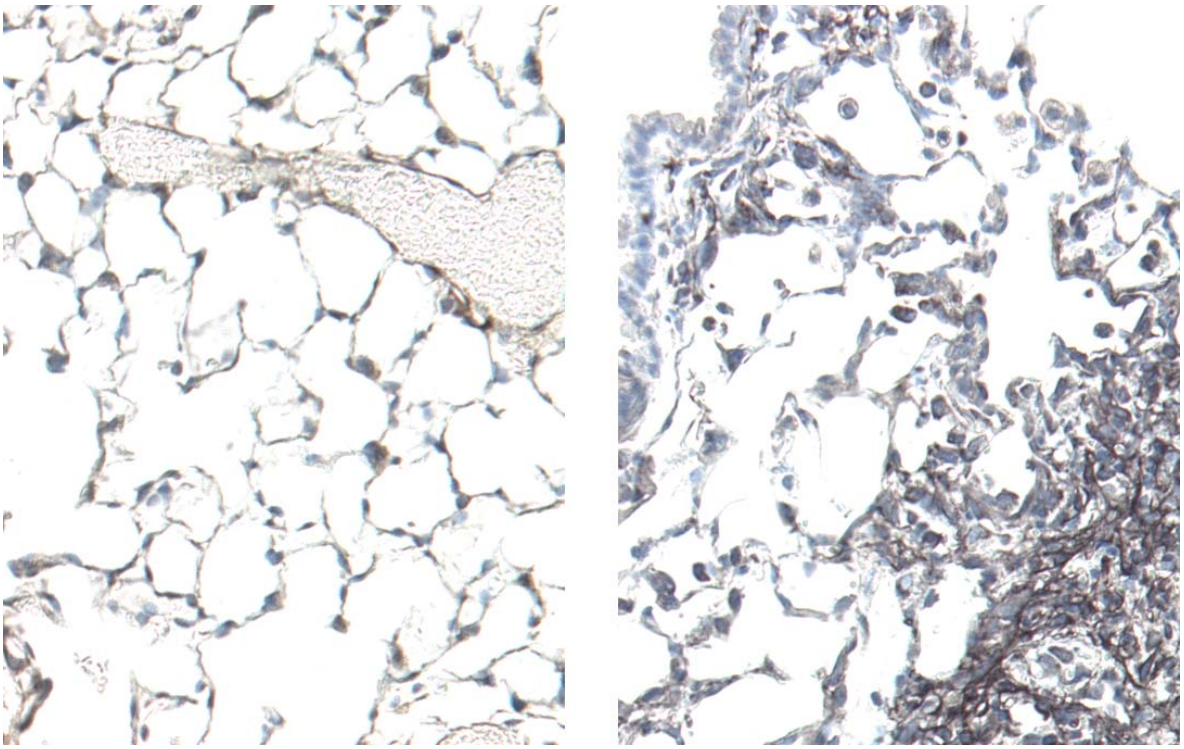
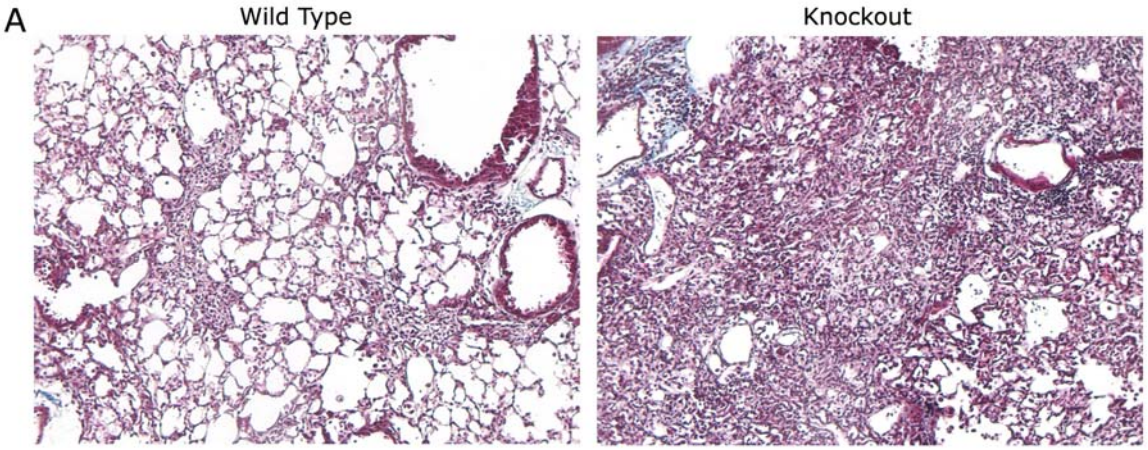


Fig. 1. Periostin Expression in Control and Fibrotic Mouse Lung Tissue. *Male, 10-week old, CD-1 mice received intraoral bleomycin or saline vehicle. After 14 days, mice were sacrificed, tissue sections were prepared, and stained using an affinity-purified polyclonal antibody prepared against a 19-amino acid peptide found in all known forms of periostin. Brown product = positive staining, the counterstain is blue. The staining pattern is described in the text.*



B

Portion of Lung With Abnormal Morphology

Mice	Treatment	Score on Arbitrary Scale 1	2	3	4
Wild Type	Saline	2	0	0	0
Knockout	Saline	2	0	0	0
Wild Type	Bleomycin	0	1	3	0
Knockout	Bleomycin	0	0	1	4

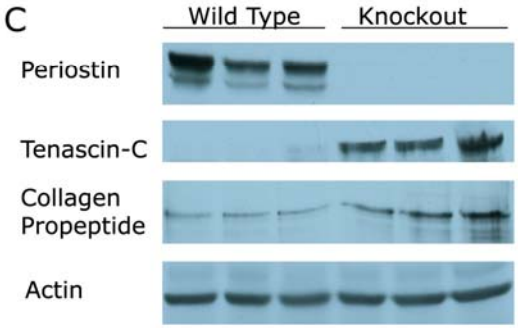


Fig. 2. Lung Injury/Fibrosis is More Severe in Periostin Knockout Mice than in Wild-Type Mice. *Periostin knockout mice and wild-type littermate mice were treated with bleomycin or saline vehicle. After 12 days, the mice were sacrificed and lung tissue sections and tissue extracts were prepared.* ***(A)*** *Representative H & E-stained tissue sections from wild type and knockout mice.* ***(B)*** *The morphology of lung tissue was scored on an arbitrary scale in which normal morphology = 1, slightly altered morphology = 2, moderately altered morphology = 3, and severely altered morphology = 4.* ***(C)*** *Lung tissue extracts were Western blotted using antibodies against Periostin (to validate the identity of knockout and wild type animals), Tenascin-C and Collagen I α1 subunit propeptide (to evaluate fibrosis), and Actin (loading control).*

KEY RESEARCH ACCOMPLISHMENTS

We have observed that:

- The C-terminal domain of periostin is a major site mediating cell to ECM adhesion
- The cell-surface receptor for the C-terminal domain is very likely to be a heparan sulfate proteoglycan
- Periostin is expressed primarily in the alveolar walls in normal lung tissue and in the distorted portion of the lung filled with ECM and inflammatory cells in fibrotic lung tissue
- Bleomycin-induced lung injury/fibrosis results in a more distorted tissue morphology in periostin knockout mice than in wild-type littermates
- Bleomycin-induced lung injury/fibrosis also results in a more severe disease in periostin knockout mice than in wild-type littermates evaluated in terms of the expression of the ECM proteins tenascin-C and collagen I α1 subunit

REPORTABLE OUTCOMES

None.

CONCLUSION

These results demonstrate the likely importance of periostin in lung injury/fibrosis. Our finding that the C-terminal domain of periostin plays a major role in cell-ECM adhesion and that its receptor is likely to be a heparan sulfate proteoglycan is novel and should lead to important discoveries on signaling pathways initiated by the binding of periostin to cells. This observation is also consistent with recent literature suggesting the importance of heparan sulfate proteoglycans in fibrosis (6, 7). The observation that the distribution of periostin is very different in control and fibrotic lung tissues highlights the fact that as we continue these studies it will be important to look at the distribution of periostin at high magnification, at a series of time points during the progression of fibrosis, and using antibodies available to us that are specific to various parts of the molecule including the C-terminus. It will also be important to evaluate which cells are expressing the periostin (e.g. fibroblasts, epithelial cells, inflammatory cells). Finally, the observation that bleomycin-induced lung injury/fibrosis produces a more severe disease in periostin knockout mice than in wild-type littermates is an unexpected result that demands careful study. In particular, we will focus on determining which cell types are differentially affected to answer questions such as: Does periostin affect the conversion of epithelial to mesenchymal cells which is believed to be one source of the fibroblasts that overexpress collagen in fibrotic lung tissue? Does periostin regulate the migration of inflammatory cells into damaged lung tissue? Does periostin negatively regulate the expression of collagen (and other ECM proteins) by lung fibroblasts, resulting in the overexpression of collagen (and other ECM proteins) in its absence? In summary, our observations open up the possibility that reagents that activate or block the signaling cascades initiated by the binding of functional sites in periostin to their receptors will have therapeutic value in inhibiting the progression of lung injury/fibrosis in human patients.

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APPENDICES

None.